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| <p>(54) Title: GRAM-NEGATIVE BACTERIAL ENDOTOXIN BLOCKING MONOCLONAL ANTIBODIES</p> <p>(57) Abstract</p> <p>Monoclonal antibodies which bind to determinants defined by the lipid A portions of the cell wall lipopolysaccharides of <i>E. coli</i> Rc mutants and <i>Salmonella</i> Re mutants and neutralize Gram-negative bacterial endotoxin, and which are chromatographically separable into two fractions by ion exchange chromatography. Human IgM embodiments of the antibodies are exemplified. The antibodies are useful for treating bacteremia or sepsis by parenteral administration.</p> | | |

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GRAM-NEGATIVE BACTERIAL ENDOTOXIN
BLOCKING MONOCLONAL ANTIBODIES

This invention is in the field of biotechnology and relates to somatic cell hybridization and immunology. More particularly, it concerns: monoclonal antibodies that bind to Gram-negative bacterial endotoxins and block the biological effects thereof; hybrid cell lines that produce the antibodies; and treatment of Gram-negative bacteremia and sepsis with the antibodies and antibiotics.

Bacteremia due to Gram-negative bacteria is a major public health problem that results in a substantial number of deaths per year. Symptoms of bacteremia include: fever, leukopenia and hypoglycemia, hypotension and shock, impaired perfusion of essential organs, activation of C5a and the complement cascade, intravascular coagulation and death.

These effects of bacteremia are attributed to the endotoxin (cell-wall lipopolysaccharide (LPS)) of the infecting organism. These LPSs are composed of three regions: Serotype-specific polysaccharide (O-antigen), core polysaccharide, and lipid A. The O-antigen region is made up of repeating oligosaccharide combinations that define type-specific haptenic determinants. The core region is composed of an outer core of hexoses (N-acetylglucosamine, glucose, galactose), and an inner core of heptose, ethanolamine and 2-keto-3-deoxyoctonate (KDO). This region is more conserved among bacterial species than the O-antigen region but does exhibit limited intraspecies and interspecies variation. Lipid A is composed of diglucosamine-4-phosphate, long chain fatty acids, and ethanolamine. KDO forms the link between lipid A and the core region.

Mutants of Gram-negative bacteria that lack the O antigen are called "rough" or "R" forms because they do not form smooth colonies on solid media. Several chemotypes of mutant LPSs are known such as Ra, Rb, Rc, Rd, and Re. In chemotype Rc the enzyme UDP-galactose-4-epimerase is deficient so that glucose, but not galactose, is synthesized and incorporated into the core. Re chemotypes lack the O and core regions up to KDO. Ziegler, E. J., et al., N. Engl. J.

Med. (1982) 307:1225-1230, describe the preparation of human antisera to the E. coli Rc mutant J5 by vaccinating healthy patients with heat-killed J5. The antisera were used to treat bacteremia and were reported to block the biological effect of LPS.

- 5 Kohler, G. and Milstein, C., Nature (1975) 256:495-497, pioneered the use of somatic cell hybridization to make continuous hybridomas that produce monoclonal antibodies. Their work used plasmacytomas and lymphocytes of murine origin. Mutharia et al., Infect. Immun. (1984) 45:631-636, and Nelles et al., Infect. Immun.
10 (1984) 46:677-681, describe LPS cross-reactive murine monoclonal antibodies.

- Subsequent investigators have reported applying Köhler and Milstein's techniques to human cells, for example, Croce, C. M., et al., Nature (Lond) (1980) 288:488 and Olsson, L. and Kaplan, H. S.,
15 PNAS (USA) (1980) 77:5429. The literature reports the preparation of human monoclonal antibodies directed against various antigens. EP 44,722 describes a mutant myeloma cell line which, after fusion with antigen-sensitized human spleen B-lymphocytes, yields monoclonal antibody-secreting human-human hybridomas. Also, it suggests making
20 antibodies to "pathogen surface antigens" and "toxins." In addition, Foug et al., J. Immun. Meth. (1984) 70:83-90, discloses human monoclonal antibody production from an EBV-transformed B cell line by fusion to a human-mouse hybridoma. Several fusion partners are described by D. Buck et al., Chapter 11 in Monoclonal Antibodies and
25 Functional Cell Lines, ed. by R. Kennett et al., Plenum Publishing Corp., 1984 and by Larrick and Buck, Biotechniques (1984) 2:6-14. Europ. Pat. Publications 107,528 and 105,804 describe cell lines capable of producing human monoclonal antibodies against a bacterial toxin. In addition, GB 2,086,937; GB 2,113,715; EP 57,107; EP 62,409;
30 EP 118,893; EP 124,301 and EP 131,878 all relate to manufacture of human monoclonal antibodies from hybrid cells.

The antibodies of the invention are characterized in that

- (a) their population is substantially homogeneous;
- (b) they bind strongly to determinants that are defined by

the lipid A of the cell-wall lipopolysaccharides of either E. coli Rc mutants or Salmonella Re mutants;

(c) they bind to either the E. coli Re mutant lipid A determinants or the Salmonella Re mutant lipid A determinants in
5 intact LPS and in whole Gram-negative bacteria; and

(d) they block the adverse biological effects of the lipopolysaccharides.

Stable, permanent hybrid cell lines that produce the above-described antibodies and progeny of those lines, and a specific mouse
10 x human B-lymphocyte fusion partner producing such cell lines and partially adapted to serum-free medium are another aspect of the invention.

The invention also contemplates compositions for treating one or more of the above-described antibodies. Preferred compositions
15 comprise two or more of the antibodies each of which binds to a different determinant located as specified in (b) above. Methods for treating bacteremia or sepsis in a human patient by administering an effective amount of such compositions to the patient are also part of the invention. Such methods for treating bacteremia include the
20 administration of antibiotics and antibody to the subject in need of treatment.

These and other aspects of the invention are described in detail below.

Figure 1 shows the growth curve of D-234 cells in serum-free
25 media HL-1 (Ventrex Labs, Portland, Me) spinner culture. The circles represent IgM levels, the triangles represent cell yield, and the squares represent glucose levels.

Figure 2 shows the growth curve of D-267 cells in serum-free media HB104 (Hana Biologicals, Berkeley, CA) spinner culture, where
30 the circles, triangles and squares are as in Figure 1.

Figure 3 shows the purification scheme for T88.

Figure 4 shows the elution profile of T8810A from a Bakerbond ABx column.

Table X presents a comparison of u chain concentrations in peaks 1 and 2.

Table XI presents a comparison of peak 1 and peak 2 LPS binding.

5 As used herein the term "cell line" refers to individual cells, harvested cells, and cultures containing cells so long as they are derived from cells of the cell line referred to.

As used herein with respect to hybrid cell lines the term "progeny" is intended to include all derivatives, issue, and offspring
10 of the cell lines regardless of generation or karyotypic identity.

As used herein the term "monoclonal antibody" refers to an antibody selected from antibodies whose population is substantially homogeneous, i.e., the individuals of the antibody population are identical except for naturally occurring mutations. Thus, the
15 antigen-binding fragment of the antibody population is the same, but the constant regions may vary.

As used herein with respect to a given monoclonal antibody the term "functional equivalent" means a monoclonal antibody that recognizes the same determinant as and crossblocks the monoclonal
20 antibody referred to. It is intended to include antibodies of the same or different immunoglobulin class and antigen binding fragments (e.g., Fab, F(ab')₂, Fv) of the monoclonal antibody.

As used herein with respect to administering antibody to patients the term "treat" and conjugates thereof refers to therapy
25 and/or prophylaxis.

As used herein with respect to characterizing the claimed hybrid cell lines the terms "permanent" and "stable" mean that the lines remain viable over a prolonged time, typically at least about six months, and maintain the ability to produce the specified
30 monoclonal antibody through at least about 25 passages.

As used herein with respect to characterizing the monoclonal antibodies herein the term "binds strongly" means that the antibody exhibits a relatively strong binding affinity to lipid A determinants of the E. coli Rc mutant LPS or Salmonella Re mutant LPS.

As used herein with respect to describing LPS, the term "intact" means that the LPS has 'O' antigen carbohydrates.

As used herein the term "whole Gram-negative bacteria" means any Gram-negative bacteria with all of its component parts, not just
5 the intact or core LPS, lipid A or rough mutant portions thereof.

As used herein the term "antibiotics" refers to a group of organic chemicals that inhibit the growth and proliferation of microorganisms, especially prokaryotic microorganisms, or that kill such microorganisms. Many of said antibiotics are the products of
10 microorganisms, especially the soil microorganisms of the Streptomyces. A number of synthetic and/or semi-synthetic antibiotics are known, e.g., amoxicillin. Of particular importance to the invention are antibiotics that have a broad spectrum of activity against Gram-negative microorganisms. Such antibiotics as are known
15 to be useful in the treatment of Gram-negative bacteremia and Gram-negative sepsis, are particularly important. Gram-negative bacteremia and sepsis is commonly treated with aminoglycoside antibiotics and, usually, at least one of the following antibiotics: cephalosporins, penicillins, beta-lactams, chloramphenicol, erythromycin, vancomycin,
20 trimethoprim sulphamethoxazole, clindamycin, rifampicin, metronidazole and quinolone antibiotics.

Monoclonal antibodies that meet the functional criteria of the invention (specific bacteria binding, endotoxin blocking) may be made using cells of diverse mammalian origin. Rat and human
25 embodiments have been made. The antibodies may be of any isotype, including IgG and IgM, with IgM types being specifically exemplified herein. The human embodiments are the products of triomas synthesized by somatic cell hybridization using a mouse x human parent hybrid cell line and Epstein-Barr virus (EBV)-transformed human PBLs or
30 splenocytes from non-immunized volunteers or volunteers immunized with available Gram-negative bacterial vaccines or inactivated Gram-negative bacteria. Fresh PBLs or splenocytes (not transformed) may be used, if desired. The rat embodiments are the products of hybridomas synthesized by somatic cell hybridization using a rat myeloma line and
35 splenocytes from rats immunized with an E. coli Rc mutant.

A preferred strategy for preparing and identifying hybrids that produce antibodies of the invention follows. Cells (PBLs, splenocytes, etc.) are panned on LPS coated tissue culture plates, then EBV transformed and fused to the tumor fusion partner (mouse myeloma x human B cell or rat myeloma). Panning involves incubation of the population of immunocompetent cells on a plastic surface coated with the relevant antigen. Antigen-specific cells adhere. Following removal of non-adherent cells, a population of cells specifically enriched for the antigen used is obtained. These cells are transformed by EBV and cultured at 10^3 cells per microtiter well using an irradiated lymphoblastoid feeder cell layer. Supernatants from the resulting lymphoblastoid cells are screened by ELISA against an E. coli Rc LPS and a Salmonella Re LPS. Cells that are positive for either Rc or Re lipid A LPS are expanded and fused to a 6-thioguanine-resistant mouse x human B cell fusion partner. If the mouse x human B cell fusion partner is used, hybrids are selected in ouabain and azaserine. Supernatants from the Rc or Re positive hybrids are assayed by ELISA against a spectrum of Gram-negative bacteria and purified Gram-negative bacterial LPSs. Cultures exhibiting a wide range of activity are chosen for in vivo LPS neutralizing activity. Many but not all antibodies so produced are of the IgM class and most demonstrate binding to a wide range of purified lipid A's or rough LPS's. The antibodies demonstrate binding to various smooth LPS's and to a range of clinical bacterial isolates by ELISA.

As used herein the term "neutralizing" is used to denote the ability of an antibody to block the adverse biological effects of Gram-negative bacteria endotoxin in vitro or in mammals regardless of the particular mechanism involved. It is intended to include, without limitation, mechanisms in which the antibody affects the biological activity of the endotoxin by binding thereto, causes the endotoxin to be degraded, or affects the activity of the endotoxin by altering the kinetics and/or site of its clearance. Neutralizing activity may be assayed in vivo using a murine model. Balb/C mice caged at 37°C , for example, may be injected i.p. or i.v. with a lethal dose of LPS (LD_{50}) at which 50% of the mice die (approximately 1-10 g). Antibody may be

injected i.p. or i.v. at 0.2 to 20 mg/kg before or after the LPS injection. The neutralizing effect is determined by comparing the morbidity of test mice with that of control mice (e.g., mice given no antibody, mice given non-binding antibody, etc.).

5 The hybridomas that produce the invention antibodies may be grown in suitable culture media such as Iscove's media or RPMI-1640 medium (Gibco, Grand Island, NY) or in vivo in immunodeficient laboratory animals. If desired, the antibody may be separated from the culture medium or body fluid, as the case may be, by conventional
10 techniques such as ammonium sulfate precipitation, ion exchange chromatography, affinity chromatography, electrophoresis, microfiltration, and ultracentrifugation.

 The monoclonal antibodies of this invention may be used passively to immunize individuals who suffer from bacteremia or sepsis
15 or who are at risk with respect to bacterial infection. Preferably a plurality of different monoclonal antibodies, each of which recognizes and binds to a distinct determinant of the cell wall LPS located interiorly of the core region are employed. In such treatment the antibody/antibodies will normally be administered parenterally (e.g.,
20 intravenously, intraarterially, intramuscularly, intraperitoneally), preferably intravenously. The dose and dosage regimen will depend upon whether the antibody/antibodies is/are being administered for therapeutic or prophylactic purposes, the patient, and the patient's history. The total amount of an antibody administered per dose will
25 typically be in the range of about 0.1 to 20 mg/kg of patient body weight, preferably 0.1 to 10 mg/kg of patient body weight.

 For parenteral administration the antibody/antibodies will be formulated in a unit dosage injectable form (solution, suspension, emulsion) in association with a pharmaceutically acceptable parenteral
30 vehicle. Such vehicles are inherently nontoxic and nontherapeutic. Examples of such vehicles are water, saline, Ringer's solution, dextrose solution, and 5% human serum albumin. Nonaqueous vehicles such as fixed oils and ethyl oleate may also be used. Liposomes may be used as carriers. The vehicle may contain minor amounts of

additives such as substances that maintain isotonicity and chemical stability, e.g., buffers and preservatives. The antibody will typically be formulated in such vehicles at a concentration of about 1.0 mg/ml to 100 mg/ml.

- 5 The various aspects of the invention are further described by the following example. This example is not intended to limit the invention in any manner. In the example, all temperatures are in degrees Celsius.

Fusion Partners

- 10 All cell lines, were maintained in Iscove's DME medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine and 5×10^{-5} M 2-mercaptoethanol. The cell lines were checked routinely for the presence of mycoplasma. For large-scale production of human monoclonal antibodies, cell lines were adapted to serum-free growth in
15 HL-1 medium obtained from Ventrex Labs, Portland, ME, and in HB104 medium obtained from Hana Biologicals, Berkeley, CA.

A. Human B Lymphocytes

- Volunteers with naturally acquired high titer serum antibodies to E. coli J5 or S. minnesota R595 core glycolipids or
20 vaccinated with a standard available typhoid injection to produce high LPS antibody titers were used as sources of peripheral blood lymphocytes. Fifty ml of venous blood was drawn from the volunteers on days 5 and 7. The blood was centrifuged, the buffy coat was harvested, and the harvested buffy coat was gradient centrifuged using
25 Ficoll/Hypaque to separate mononuclear cells from lymphocytes. Alternatively, peripheral blood was gradient centrifuged using Ficoll/Hypaque for the same purpose. Ficoll/Hypaque in a 1 liter quantity is prepared by dissolving 64 g of Ficoll (Sigma) in 600 ml of distilled water using a stir bar rotor at low speed and then adding 99
30 g of diatrizoate sodium (Sterling Drug, N.Y.). After both substances were dissolved, more water was added to 1 liter volume and 0.7 g NaCl was added. Preferably, monocytes were depleted by adherence to

plastic. T cells were depleted by aminoethylthiuronium-treated sheep red blood cells (AET-SRBC) rosetting, using the technique described by Madsen and Johnsen, J. Immunol. Meth. (1979) 27:61-74. The remaining B cell-enriched lymphocyte population was transformed with Epstein-
5 Barr virus using the technique described by Fount et al., J. Immunol. Meth. (1984) 70:83-90, except that cells were generally cultured at 10^3 - 10^4 cells/well in 96-well culture plates.

The cultures were assayed after 20 days by ELISA using E. coli J5 as antigen (described below). The cells in one well
10 exhibiting a high antibody titer were subcultured at 1000 cells/well, then at 500 cells/well, and positive cultures containing core-specific, antibody-secreting cells were pooled.

Alternatively, in a preferred embodiment, the mononuclear cells were isolated from the peripheral blood and were incubated on
15 plastic to deplete plastic-adherent monocytes. The remaining cells (T and B) were panned on plates coated with LPS of S. minnesota Re R595 obtained from Ribi ImmunoChemResearch, Inc. Adherent cells were then transformed by EBV and maintained in culture or cultured in microtiter plates and screened for those positive for Re LPS. Those cells
20 maintained in culture were panned again ten days later on LPS of E. coli J5 from Ribi. These cells also were then cultured in microtiter plates. Panning on a specific antigen can at least double the number of recoverable antigen-specific cells and can improve the secretion rate of antibody.

25 B. Rat Splenocytes

E. coli Rc mutant J5 in saline at 10^9 cells/ml was used as antigen. Rats were injected i.p. and sq with 0.5 ml of bacterial suspension + 0.5 ml of complete Freund's adjuvant (CFA). The rats were boosted on day 25 with an identical injection and on day 29 with
30 0.2 ml of bacterial suspension injection i.v. Splenectomies were carried out on day 32.

C. F3B6 (Mouse x Human Line)

A mouse-human heterohybrid fusion partner designated F3B6 (adapted to 99% serum-free medium and deposited with the ATCC under ATCC Accession No. HB8785 on April 18, 1985) was constructed by fusing
5 human peripheral blood lymphocyte (PBL) B cells obtained from a blood bank with the murine plasmacytoma cell line NS1 obtained from ATCC under ATCC No. TIB18(P3/NS1/1-AG4-1). The PBL cells from random buffy coat were transferred to a 50 ml centrifuge tube and diluted with 30 ml Hanks' balanced salt solution (Ca^{2+} -free/ Mg^{2+} -free) (HBSS-/-).
10 Then 10 ml Ficoll-Hypaque was added and the mixture centrifuged at 1500 rpm for 15 minutes at room temperature. The interface was removed and the mixture was washed with HBSS-/- and resuspended in HBSS-/- . The cells were counted.

The NS-1 cells were grown in 4 x T75 flasks and harvested,
15 washed with HBSS-/- and resuspended in HBSS-/- . The cells were counted.

Approximately 5×10^7 B-cells and 2.5×10^7 NS-1 cells (2:1 ratio) were added to each of 5-50 ml centrifuge tubes for fusion. The mixture was centrifuged at 1200 rpm for eight minutes at room
20 temperature to form a tight pellet. All of the supernatant was removed and the tube was kept at 37°C for further manipulations. A total of 1 ml of warm 50% polyethyleneglycol of molecular weight 1540 (PEG 1540) (BDH Chemicals, Poole, England) was added over a one minute period using a 1 ml pipette. The cell pellet was gently stirred with
25 the tip of the pipette as the PEG was being added. Then 1 ml of HBSS was added at 37°C over a one-minute period to dilute gradually the PEG. The cells were washed twice with HBSS-/- and resuspended in Iscove's medium in several T150 flasks.

On day 2 the cells were washed in HBSS-/- in 50 ml
30 centrifuge tubes. A total of 10 ml of Ficoll-Hypaque was added to the tubes. The tubes were centrifuged at 1500 rpm for 15 minutes at room temperature and the live cells at the interface were removed. The pellet was washed twice with RPMI-1640 (Gibco) and resuspended in an enriched hypoxanthine/azaserine selection medium (EHA) consisting of

100 μ M hypoxanthine (Sigma), 5 μ g/ml azaserine (Sigma) and Iscove's medium (Gibco), 10% NCTC (M. A. Biologicals), 20% heat-inactivated-FBS. The density was adjusted to 2.5×10^4 cells/ml medium.

At day 5 the suspensions were washed twice with HBSS-/- and resuspended in 10 ml Iscove's medium. Live cells were separated by Ficoll-Hypaque density gradient centrifugation as described above. Cells were washed twice with RPMI-1640 + 20% FBS, and then plated out in 96-well plates at 10^6 cells/ml. At days 7, 9 and 12 the EHA selection medium described above was added each time. At days 15 and 18 the plates were fed with EHMT medium containing hypoxanthine, methotrexate and thymidine. The supernatants were assayed for Ig secretion and Ig secreting hybrid cell lines were cloned by limiting dilution in U bottom 96 well plates.

Well F3B6 was selected for 6-thioguanine selection. Several roller bottles of F3B6 were grown up. A total of 10 μ g/ml of 6-thioguanine was added to the roller bottles. Dead cells were removed by Ficoll-Hypaque density gradient centrifugation on days 2, 5 and 7. A 6-thioguanine resistant clone was grown up. Test fusions were performed, and the cell line was tested for ouabain resistance.

The resultant cell line was adapted to growth and maintenance in 99% serum-free medium and 1% FBS for more reproducible manufacturing by the following multi-step process:

1. Two days prior to subculturing, the cells were fed with a mixture of the Iscove's DME in which they were growing, 50% of the amount of FBS in the medium in which they were growing, and 50% by weight of serum-free medium HL-1 supplied by Ventrex, Inc.

2. Two days later, or when the hybridoma cells reached densities of 8×10^5 to 1×10^6 cells/ml, the cells were subcultured and planted with 50% of Iscove's DME medium and 50% of the serum-free medium. The cells were removed from the latter medium by centrifugation at $200 \times g$ for five minutes. The Iscove's DME medium was mixed with 50% of the serum-free medium to form a 50:50 mixture, in which the cell pellet was suspended and then counted. An appropriate amount of cell suspension was planted in the vessel with

50% Iscove's DME and 50% serum-free medium. The planted cell densities preferably do not fall below 5×10^4 cells/ml and not exceed 1×10^5 cells/ml.

3. After two to three days post-planting, or when the cell density reached 8×10^5 to 1×10^6 cells/ml, the cells were refed with 50% Iscove's DME and 50% serum-free medium.

4. Step 3 was repeated for another passage.

5. After two to three days in culture or when the cell density reached 8×10^5 to 1×10^6 cells/ml and viability was about 85%, the cells were cultured on serum-free medium only. When the cells were planted in the serum-free medium for the first time the cell densities were between 1×10^5 to $8-9 \times 10^5$ cells/ml. The final medium was HL1 with 1% FBS.

Rat Tumor Line

15 An available rat tumor line was used.

Fusion Protocol

The fusion mixture contained polyethylene glycol (PEG) 4000, 40% (w/v); dimethylsulfoxide (DMSO), 10% (v/v) in Hanks' balanced salt solution (HBSS)-/+ (Ca^{2+} -free, 2 mM MgSO_4). Forty g of PEG 4000 was combined with 10 ml of DMSO and 50 ml of HBSS-/. The mix was autoclaved for 25 minutes. Before use, the pH of the fusion mixture was adjusted to between 7.5 and 8.5 with sterile 0.1 N NaOH.

Plates (6-well cluster, 35 mm well diameter) were prepared as follows: 2 ml of HBSS-/+ and 50 μ l of a filter sterilized, 20-100 μ g/ml, peanut agglutinin (PNA, Sigma) were added to each well. Plates were incubated at 37°C for at least one hour prior to use. PNA stock was stored frozen, and a freshly thawed aliquot was used to coat fusion cells. Smaller sized wells were used if cell numbers were limited.

30 Parent cells in Ficoll-Hypaque were washed twice in HBSS-/+ at room temperature and subsequently resuspended and combined at a

ratio of 1:1 lymphocyte-splenocyte:fusion partner in HBSS-/+.warmed to 37°C. Two ml of the combined cell suspension ($1-3 \times 10^7$ cells/well) was added to each pretreated well containing 1 µg/ml PNA coating solution. The wells were incubated at 37°C for one minute. Plates 5 were spun onto bottom of the plate at 400-500 x g, room temperature, for five-six minutes to form a monolayer of cells. Supernatant was then aspirated off the plates, leaving behind adherent coating of cells.

Two ml of PEG fusion mixture described above and warmed to 10 37°C was carefully added down the side of the fusion cell. After one minute, the PEG solution was diluted with a fusion dilution mixture (FDM) of 5% DMSO (Sigma) HBSS-/+ (warmed to 37°C and filter sterilized) at a rate of 2 ml/min (0.5 ml every 15 sec) for the next two-three min (4-6 ml). For the next two minutes the FDM was added at a rate of 4 15 ml/min with mixing. FDM was always added down the side of the well, so as not to disturb the monolayer, and the plates were swirled constantly to ensure optimal mixing.

At the end of the two minutes the wells were aspirated to remove diluted PEG fusion mixture. The remaining film of PEG mixture 20 was diluted at a rate of 2 ml/min for two min with warm FDM. Again the plate was constantly swirled. Over a period of 0.25-2 minutes with swirling, 5 ml of HBSS-/+ warmed to 37°C was added to the fusion well at a rate of 1 ml/15 sec. The well was then filled up with HBSS-/ + and all supernatant was aspirated from the monolayer. Finally, 25 each fusion well was washed once or twice with about 5-10 ml of warm HBSS-/ + and aspirated. Five ml of warm Iscove's complete medium and 15-20% FCS, were added to each well, and the plates were incubated at 37°C for 24 hours. The day following fusion the cells were resuspended at a density of 5×10^5 cells/ml in EHA medium containing 30 azaserine (2 µg/ml), hypoxanthine (100 µM), and ouabain (1 µM) and plated at 200 µl or well in 96-well plates. Cultures were subsequently fed every three days. Growing hybrids were visible by day 10. Preferably, after selection was completed, azaserine and ouabain were gradually weaned out of serum then hypoxanthine as well.

ELISAsA. Bacteria

Immulon microtiter plates (Dynatech) were used or plates were prepared as follows for use in ELISAs. Fifty-100 μ l of 0.5-1.0% glutaraldehyde (Sigma) in deionized water was coated onto flat/bottom microtiter plates (Dynatech). After one to four hours of incubation at room temperature, the wells were aspirated or washed twice with distilled water.

Bacteria were grown overnight, spun down, washed in saline three times and reconstituted to .25% (v/v). One-hundred μ l of this bacteria suspension was used to coat each well of a 96-well flat bottom Immulon microtiter plate or plates prepared as above. Plates were spun for 20 minutes at 2000 rpm. The suspensions were incubated overnight or for a minimum of two hours. The plates were then washed either overnight with 100 ml volume or three times on automatic plate washer with phosphate buffered saline containing 0.1 g/l MgSO_4 and 0.1 g/liter CaCl_2 (PBS^{++}), .05% Tween 20 surfactant (Sigma) and .01% thimerosol. To decrease background, plates could be baked for one hour at 37°C with PBS + 1% BSA. (Bovine serum albumin - Sigma) supernatant was incubated for 30-60 or 90 minutes at room temperature and washed 3 times with PBS^{++} , Tween 20 and thimerosol. Fifty-100 μ l of horseradish peroxidase (HRP) conjugated goat anti-human IgG (Tago, Inc.) or HRP conjugate goat anti-human IgM (Jackson Labs) was added to each well. Plates were incubated at room temperature 40°C for 30 minutes and washed 5 times with PBS^{++} , .05% Tween 20 and .01% thimerosol. Two-hundred μ l of ABTS substrate was then added to each well, the substrate consisting of 55 mg (ml of ABTS aqueous stock solution diluted 1:1000 with .1 M sodium citrate buffer pH 4.5 to which 1:1000 of 30% H_2O_2 was added immediately at 37°C in the dark. The contents of the wells were transferred to a transparent plate for reading or were read directly with an ELISA reader at 405 nm. readings reported on a scale of 1-10 with 1=.2 OD and 1.0=2.0 OD.

B. LPS

Flat-bottom microtiter plates were coated overnight with 50 μ l of a preparation of sonicated LPS, 50 μ g/ml in 0.05 mM sodium bicarbonate buffer, pH 9.6. Plates were washed with PBS⁺⁺ with 0.05% Tween 20 (Sigma), and preferably 0.01% thimerosal up to five times by immersion or with an automated plate washer. Subsequently, 100 μ l of PBS⁺⁺, 1% BSA, 0.05% Tween 20, and preferably 0.01% thimerosal was added to each well, followed by 100 μ l of the test supernatants. Supernatants were incubated for 30 minutes at 4°C to room temperature and then washed up to five times with the PBS⁺⁺/Tween/thimerosal mixture. Then a total of 50-100 μ l of peroxidase-conjugated goat anti-human IgM (Tago) diluted in PBS⁺⁺, BSA, Tween 20 and thimerosal was added and the mixture incubated for 30 minutes at room temperature or 40°C and washed up to five times. Then 200 μ l of the ABTS peroxidase substrate described for the bacterial ELISA was added to each well and each well was incubated for 30 minutes at 37°C in the dark. The contents of the wells were read on a plate ELISA reader at 405 nm.

C. IgM

Immulon II flat-bottom microtiter plates were coated at 100 μ l/well with goat anti-human IgM (Tago) diluted 1:100 in 50 mM bicarbonate buffer (pH 9.6). After 90 minutes at 37°C, plates were washed with PBS⁺⁺, 0.05% Tween 20, and preferably 0.01% thimerosal up to five times by immersion or with automated plate washer. Then 100 μ l of PBS⁺⁺, 1% BSA, 0.05% Tween 20, 0.01% thimerosal was preferably added to each well. A total of 100 μ l of test supernatant was added to first wells and preferably duplicate two-fold dilutions were made. One well was preferably left as control. The plates were incubated for 30 minutes at 22°C and then washed up to five times as described above. Then, a total of 50-100 μ l of peroxidase-conjugated goat anti-human IgM antibody (Tago) diluted in PBS⁺⁺, BSA, Tween 20 and thimerosal was added and the mixture incubated for 30 minutes at room temperature or 40°C and washed up to five times. Then a total of 200 μ l of the ABTS peroxidase substrate described for the bacterial

ELISA was added to each well. The mixture was incubated for 30 minutes at 37°C in the dark and read on an ELISA plate reader (OD₄₀₅) using as IgM standard pooled human myeloma (Cappell) previously standardized versus a Tago Standard.

5 Hybrid Screening

A. B Lymphocyte x F386

Culture supernatants were assayed by ELISA as described above using commercial E. coli J5 and S. minnesota Re595 LPS's (obtained from List Biologicals or Ribi ImmunoChemResearch, Inc.).
10 Positive wells were subcloned by limiting dilution and reassayed approximately two weeks later. Limiting dilution cloning was performed in 96-well U-bottom plates in Iscove's DME medium with 20% FCS.

Selection for a nonproducer parent cell line or a high-
15 producer hybrid was accomplished using a reverse-plaque technique. Protein A-coated sheep erythrocytes (1.0%) were added to the upper layer of soft agar according to the method of Gronowicz et al., Eur. J. Immunol. (1976) 6:588-590.

Sixteen hybrids were chosen based on their titers of anti-Re
20 and anti-J5 antibody for expansion and further testing.

Sixteen human monoclonal antibodies produced from these hybrids were isotyped using the IgM ELISA mentioned above and all were of the IgM class. All hybrids have been cloned and stably produce greater than 10 µg of antibody per ml of spent culture media. Table I
25 presents the ELISA results of these anti-LPS hybridomas binding to various purified core lipopolysaccharides commercially obtainable from Ribi ImmunoChemResearch, Inc. D253 is a negative control human monoclonal antibody which does not bind LPS but binds P. aeruginosa toxin A. Table II demonstrates binding of these monoclonal antibodies
30 to various rough mutant bacteria commercially obtainable from Ribi ImmunoChemResearch, Inc. Table III demonstrates binding of these same antibodies to a variety of bacteremic Gram-negative clinical isolates.

This series of sixteen antibodies shows a number of different binding patterns:

1. Some of the antibodies recognize only core antigenic determinants;
2. Some recognize core antigenic determinants as well as determinants found on certain smooth lipopolysaccharide molecules; and
3. Some antibodies demonstrate broad cross-reactivity to not only core antigenic determinants, but to O-antigenic determinants found on whole bacteria.

TABLE I

CORE LIPOPOLYSACCHARIDES

| | Anti-body | Salmonella | | | | | |
|----|-----------|---------------------------|---------------------------|-----------------------|-----------------------------------|--------------------------------------|----------------------------|
| | | <u>E. coli</u> J5 (Rc) | <u>minn.</u> R595 (Re) | <u>S. typhi</u> Re | <u>E. coli</u> J5 (Rc) Lipid A | <u>S. minn.</u> R595 (Re) Lipid A | <u>S. typhi</u> Lipid A |
| 15 | D253* | 0** | 1 | 1 | 0 | 1 | 1 |
| | D234 | 7 | 7 | 4 | 7 | 1 | 9 |
| | D267 | 3 | 6 | 1 | 3 | 1 | 7 |
| | D250 | + | | | | | |
| | D244 | + | + | | | | |
| 20 | L116 | 8 | 9 | 0 | 8 | 9 | 8 |
| | L118 | 8 | + | 2 | 9 | 9 | + |
| | L119 | 9 | + | 6 | + | 9 | + |
| | L121 | 9 | 8 | 9 | 9 | 8 | + |
| | L123 | 8 | 9 | 8 | 9 | 9 | 9 |
| | L124 | 0 | 3 | 0 | 1 | 3 | 1 |
| | L126 | 0 | 7 | 0 | 3 | 8 | 0 |
| 30 | WI-3 | 8 | + | 3 | + | 9 | 9 |
| | WI-4 | 3 | + | 0 | + | 9 | + |
| | WI-5 | 4 | 9 | 1 | 8 | 8 | 7 |
| | WI-6 | 6 | + | 0 | 9 | + | + |
| | WI-7 | 7 | 8 | 0 | + | + | + |

* Negative control

** Magnitude of numbers indicates the degree of monoclonal binding.
0 is negative, + is off scale with the plate reader set at 2.0
at 2.0 absorbance full scale.

TABLE II

ROUGH MUTANT - BACTERIA

| Antibody | SL3770† | SL3749 | SL3750 | SL3748 | E. coli | | S. minn. R595 (Re) |
|----------|---------|--------|--------|--------|---------|--------|-----------------------|
| | | | | | J5 (Rc) | SL3789 | |
| D253* | 0** | 0 | 0 | 0 | 0 | 0 | 0 |
| D234 | 7 | + | + | + | + | + | + |
| D267 | 1 | 4 | 5 | 7 | 6 | 3 | 7 |
| D250 | ND | ND | ND | ND | 9 | ND | 0 |
| D244 | 0 | 1 | 0 | 3 | 5 | 1 | 8 |
| <hr/> | | | | | | | |
| L116 | 0 | 0 | 0 | 5 | 0 | 0 | 7 |
| L118 | 0 | 3 | 5 | 9 | 4 | 4 | + |
| L119 | 0 | 0 | 0 | + | 2 | 0 | + |
| L121 | 0 | 0 | 1 | + | + | 0 | + |
| L123 | 0 | 1 | + | + | 9 | 1 | + |
| L124 | 0 | 0 | 0 | 1 | 0 | 0 | 1 |
| L126 | 0 | 0 | 0 | 2 | 0 | 1 | 5 |
| <hr/> | | | | | | | |
| WI-3 | 2 | 5 | 7 | 8 | 8 | 4 | 1 |
| WI-4 | 1 | 2 | 2 | 3 | 2 | 1 | 1 |
| WI-5 | 1 | 1 | 2 | 3 | 2 | 1 | 1 |
| WI-5 | 1 | 1 | 2 | 2 | 2 | 1 | 1 |

† See Lyman et al. (1976) *Infect. Immun.* 13:1539-1542; SL3770 is smooth; SL3749 and SL3750 are superficial rough (Ra, Rb); SL3748 is Rc and SL3789 is Rc.

* Negative control

** See legend Table I

ND = not determined

TABLE III
CLINICAL ISOLATES

| Anti-body | K. pneumoniae | | P. aeruginosa | | P. aeruginosa | | E. coli | | E. coli | | E. coli | | E. aerogenes | |
|-----------|---------------|-----|---------------|--------|---------------|----------|---------|------|---------|------|---------|------|--------------|---|
| | SM-1 | 0** | Type 1 | Type 1 | Type 111 | Type 111 | SM-1 | SM-1 | SM-2 | SM-2 | SM-5 | SM-5 | | |
| D253* | 0 | 0 | ND | 0 | 0 | 0 | 0 | ND | 0 | 0 | 0 | 0 | 0 | 0 |
| D234 | 2 | 1 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 3 | 3 | 0 | 3 | 0 |
| D267 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| D250 | 0 | 0 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 |
| D244 | 0 | 0 | 0 | ND | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| L116 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| L118 | 7 | 0 | 0 | 0 | 0 | 8 | 0 | 3 | 0 | 8 | 8 | 0 | 8 | 0 |
| L119 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| L121 | 2 | 0 | 0 | 0 | 0 | 5 | 0 | 0 | 0 | 1 | 1 | 5 | 5 | 0 |
| L123 | 1 | 0 | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 2 | 2 | 3 | 3 | 0 |
| L124 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| L126 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| WI-3 | 7 | 7 | 7 | 1 | 1 | 3 | 3 | 4 | 4 | 5 | 5 | 6 | 6 | 0 |
| WI-4 | 4 | 7 | 7 | 1 | 1 | 4 | 4 | 3 | 3 | 4 | 4 | 5 | 5 | 0 |
| WI-5 | 3 | 5 | 5 | 0 | 0 | 3 | 3 | 1 | 1 | 2 | 2 | 4 | 4 | 0 |
| WI-6 | + | 9 | 9 | 1 | 1 | 9 | 9 | 7 | 7 | 8 | 8 | 9 | 9 | 0 |
| WI-7 | 4 | 5 | 5 | ND | ND | 3 | 3 | 1 | 1 | 3 | 3 | 3 | 3 | 0 |

* Negative control

** See legend Table I

Antibody D250 binds only to J5 E. coli (Rc) core determinants with minimal binding to other core lipopolysaccharides and rough mutant bacteria. It gives spotty binding to clinical isolates of E. coli. Antibody D244 binds to E. coli J5 and Salmonella
5 minnesota R595 LPS and bacteria, but not to any other bacteria or lipopolysaccharides. Antibodies D234 and D267 show considerable binding to rough lipopolysaccharides and lipid A's with spotty binding on clinical isolates. The 'L' series and 'W' series of antibodies show high binding on rough lipopolysaccharides with a few 'holes' in
10 the binding patterns. Antibodies within the 'L' series bind to more clinical isolates with a higher binding affinity. In general, those monoclonals showing the highest amount of core LPS or rough bacterial binding demonstrate the most cross-reactivity on clinical isolates.

In another test certain clones of the 'L' and 'D' series of
15 antibodies, the D253 clone control and an antibody S261 were evaluated for their binding to purified core LPS and whole bacteria using the bacteria ELISA test described above. The results of the evaluations using purified core LPS and whole bacteria are provided in Tables IV and V for purified LPS and VI for whole bacteria.

TABLE IV

CORE LIPOPOLYSACCHARIDES

| Antibody Clone No. | E. coli | | S. minn. | | S. typhi | | E. coli | | S. minn. | | S. typhi | | E. coli | |
|-----------------------|---------|--------|----------|--------|----------|--------|------------|------------|------------|------------|----------|--------|------------|---------|
| | Rc LPS | Re LPS | Rc LPS | Re LPS | Rc LPS | Re LPS | Rc Lipid A | Re Lipid A | Rc Lipid A | Re Lipid A | Lipid A | Parent | 011:84 LPS | Plastic |
| 0253-15-6* | 0** | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 0 |
| 0234-4-27 | 7 | 7 | 4 | 4 | 4 | 4 | 7 | 7 | 1 | 1 | 9+ | 9+ | 1 | 0 |
| 0267-22-37 | 3 | 6 | 1 | 1 | 1 | 1 | 3 | 3 | 1 | 1 | 7 | 7 | 1 | 0 |
| 0250-12-4-3 | 7 | 1 | 1 | 7 | 7 | 7 | 3 | 3 | 0 | 0 | 8 | 8 | 0 | 0 |
| L116-2-4 | 2 | 7 | 7 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 7 | 7 | 0 | 0 |
| L118-8-4 | 0 | 9+ | 9+ | 0 | 0 | 0 | 9 | 9 | 2 | 2 | 5 | 5 | 0 | 0 |
| L118-22-2 | 2 | 9+ | 9+ | 0 | 0 | 0 | 9+ | 9+ | 2 | 2 | 9+ | 9+ | 0 | 0 |
| L119-4 | 8 | 8 | 8 | 2 | 2 | 2 | 2 | 2 | 1 | 1 | 8 | 8 | 0 | 0 |
| L120-1 | 3 | 6 | 6 | 1 | 1 | 1 | 3 | 3 | 9 | 9 | 5 | 5 | 9+ | 2 |
| L121-7 | 9+ | 9+ | 9+ | 9+ | 9+ | 9+ | 9+ | 9+ | 2 | 2 | 9+ | 9+ | 0 | 0 |
| L123-15 | 7 | 8 | 8 | 6 | 6 | 6 | 8 | 8 | 1 | 1 | 9 | 9 | 0 | 0 |
| L124-3 | 1 | 9+ | 9+ | 0 | 0 | 0 | 9+ | 9+ | 1 | 1 | 9+ | 9+ | 0 | 0 |
| L126-1 | 0 | 7 | 7 | 0 | 0 | 0 | 6 | 6 | 1 | 1 | 3 | 3 | 0 | 0 |
| S261*** | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 |

* Negative control

** Magnitude of numbers indicates the degree of monoclonal binding.

0 is negative and 9+ is the strongest binding with the plate reader set at 2.0 at 2.0 absorbance full scale.

*** Antibody of U.S. Serial No. 550,261 filed November 8, 1983.

In another experiment, antibody D234 and comparative antibody S261 were tested against various purified core LPS. The results are provided in Table V.

TABLE V

5

CORE LIPOPOLYSACCHARIDES

| Antibody | <u>E. coli</u> <u>Rc LPS</u> | <u>S. minn.</u> <u>Re LPS</u> | <u>E. coli</u> <u>Rc Lipid A</u> | <u>S. minn.</u> <u>Re Lipid A</u> |
|----------|---------------------------------|----------------------------------|-------------------------------------|--------------------------------------|
| D234 | 7 | -* | - | - |
| S261 | 1 | 1 | 1 | 2 |

- 10 * Magnitude of numbers indicates the degree of monoclonal binding, where the - indicates the strongest binding, which exceeds 9 on the scale.

The antibodies D234 and S261 were tested against the whole bacteria E. coli J5 and S. minnesota Re 595 using the bacterial ELISA
15 test described above. The results are indicated in Table VI.

TABLE VI

WHOLE BACTERIA

| Antibody | <u>E. coli</u> <u>Rc LPS</u> | <u>S. minn.</u> <u>Re LPS</u> |
|----------|---------------------------------|----------------------------------|
| 20 D234 | -* | - |
| S261 | - | - |

* See legend of Table V

The results in Tables I-VI indicate that all of the monoclonal antibodies produced in accordance with this invention,
25 except L116-2-4, which is not part of the invention in view of its properties, meet the binding criteria defined herein, i.e., they bind strongly to the purified lipid A determinants of E. coli Rc and/or S. minn. Re LPS (Tables I, IV and V) and they bind to these determinants in intact LPS (Table III) and in whole bacteria (Table VI). In
30 contrast, Tables IV and V show that the S261 comparative antibody does not bind or binds very weakly (one-seventh the strength of D234) to the purified lipid A determinants of E. coli Rc and/or S. minn. Re.

In a neutralization experiment, mice were injected intravenously with a dose of 0.2 ml each of a 1:1 dilution of a hybridoma culture supernatant of negative control antibody D-253 or invention antibody D250, described above. Four hours later the mice were challenged i.p. with 7, 10-fold dilutions of E. coli J5 core LPS bacteria, five mice per dilution, where before infection the bacteria were suspended in 0.5 ml of hog gastric mucin, 5% in normal saline, to which 15 mg of D-galactosamine had been added. Table VII indicates the results, where LD₅₀ is the lethal dose of LPS bacteria at which 50% of the mice die.

TABLE VII

| Monoclonal Antibody | LD ₅₀ | Fold Increase in LD ₅₀ compared to control |
|------------------------|-----------------------|--|
| D253 (control) | 9.8 x 10 ⁴ | -- |
| 15 D250 | 2.5 x 10 ⁶ | 26 |

The results show that the D250 antibody of this invention exhibits statistically significantly more protective activity in mice against the endotoxin core determinants of E. coli J5 than the negative control antibody D253, indicating the neutralizing or blocking property of an invention antibody.

Two of the hybridomas (D234 and D267) were adapted to growth and maintenance in serum-free medium for large-scale, more reproducible spinner culture production of monoclonal antibodies using the following step-wise method:

1. Two days prior to subculturing, the cells were fed with a mixture of the Iscove's DME in which they were growing, 50% of the amount of FBS in the medium in which they were growing, and 50% by weight of serum-free medium HL-1 supplied by Ventrex, Inc. or HB104 supplied by Hana Biologicals.

2. Two days later, or when the hybridoma cells reached densities of 8 x 10⁵ to 1 x 10⁶ cells/ml, the cells were subcultured and planted with 50% of Iscove's DME medium and 50% of the serum-free medium. The cells were removed from the latter medium by

centrifugation at 200 x g for five minutes. The Iscove's DME medium was mixed with 50% of the serum-free medium to form a 50:50 mixture, in which the cell pellet was suspended and then counted. An appropriate amount of cell suspension was planted in the vessel with 5 50% Iscove's DME and 50% serum-free medium. The planted cell densities preferably do not fall below 5×10^4 cells/ml and not exceed 1×10^5 cells/ml.

3. After two to three days post-planting, or when the cell density reached 8×10^5 to 1×10^6 cells/ml, the cells were refed with 10 50% Iscove's DME and 50% serum-free medium.

4. Step 3 was repeated for another passage.

5. After two to three days in culture or when the cell density reached 8×10^5 to 1×10^6 cells/ml and viability was about 85%, the cells were cultured on serum-free medium only. When the 15 cells were planted in the serum-free medium for the first time the cell densities were between 1×10^5 to $8-9 \times 10^5$ cells/ml.

Figure 1 shows the growth curve of D-234 cells in serum-free medium HL-1 (Ventrex). Figure 2 shows the growth curve of D-267 cells in serum-free medium HB104 (Hana Biologicals). Under these 20 conditions, as much as 50-75 μ g/ml of specific human monoclonal antibody was produced.

B. Splenocyte xF3B6 Fusion

Production of T88

A monoclonal antibody producing hybridoma designated T88 was 25 obtained as the fusion product of human splenocytes with cell line F3B6.

A human spleen specimen from a lymphoma patient was mascerated in Hank's balanced salt solution to release lymphocytes from the parenchyma of the spleen and suspend the cells. The 30 suspended cell fraction was collected and was gradient centrifuged using Ficoll/Hypaque to separate lymphocytes. T cells were removed by aminoethylthiouronium treated sheep red blood cells (AET-SRBC) rosetting, using the technique described by Madsen and Johnsen, J. Immunol. Meth. (1979) 27:61-74.

The remaining cells were panned on plates coated with LPS of S. minnesota Re R595 obtained from Ribi ImmunoChemResearch, Inc. Adherent cells enriched in B-cell population were transformed with Epstein-Barr virus using the technique described by Fount et al., J. Immunol. Meth. (1984) 70:83-90, except that cells were generally cultured at 10^3 - 10^4 cells/well in 96-well culture plates.

The cells were maintained in culture and assayed after 20 days by ELISA using E. coli J5 as antigen (described above). The cells in one well exhibiting a high antibody titer were subcultured at 1000 cells/well, then at 500 cells/well, and positive cultures containing core-specific, antibody-secreting cells were retained for fusion.

Fusions were carried out with cell line F386 using 40% (w/v) PEG 4000; DMSO, 10% (v/v) and 5 μ g/ml poly I arginine in Hank's balanced salt solution (HBSS)-/+ (Ca^{2+} -free, 2 mM MgSO_4) using the fusion protocol described hereinabove. Hybrids produced by the fusion were selected using azaserine, hypoxanthine and ouabain resistance and assayed by ELISA on LPS on whole J5 bacteria (described hereinabove). T88 was identified as a cell producing antibody reactive with LPS antigen and has been deposited under accession number CTCC 10235 in applicant's depository. It has been accepted for deposit in the American Type Culture Collection under the terms of the Budapest Treaty for the deposit of microorganisms for patent purposes and has accession number HB7431.

25 Therapeutic Effects of Human Anti-J5 Monoclonal Antibodies

I. E. coli SM18 Sepsis Model

A. General Methods

In these examples, outbred CD-1 female mice weighing 23-25 grams were injected intraperitoneally (i.p.) with 6×10^7 colony forming units of E. coli strain SM18. Thirty minutes after administration of the bacteria, antibody in 0.5 ml PBS was injected intravenously (i.v.) in the tail vein of experimental mice. One and three hours after administration of the bacteria 3.2 mg/kg gentamicin

was injected intramuscularly (i.m.) in each mouse using 0.05 ml saline as a carrier. Type-specific polyclonal rabbit antibody to SM18 and an irrelevant (IgM) antibody (Rockland) were run as controls in some experiments as indicated. In all experiments, a bacterial control was run in which the mice were sham injected with PBS i.v. and i.m.

B. Results Using D234

D234 was administered as indicated above in four separate experiments. In two experiments, VIII-1 and VIII-2 below, polyclonal type-specific rabbit immune globulins to E. coli strain SM18 was also used as a positive control. In two experiments, VIII-3 and VIII-4, myeloma human IgM was used as a negative non-specific antibody control.

The results of each are shown in Tables VIII-1 through VIII-4. The data from the four experiments were compiled and the results are presented in Table VIII-5 as the net increase in survival (%) of antibody treated groups over that of gentamicin alone treated groups. As shown in Table VIII-5, when administered at 10 mg/mouse (363 mg/kg), D234 was protective against experimental E. coli in mice. At the lower doses of 5 mg/mouse (182 mg/kg) and 2.5 mg/mouse (91 mg/kg), significantly enhanced survival time was observed. However, protective effect was not observed when D234 was administered at 1.0 mg/mouse (36 mg/kg) in the same mouse model. The non-specific control antibody (Rockland IgM) has not shown significant protective effect when tested at 14 mg per mouse (500 mg/kg) and at 4 mg/mouse (200 mg/kg).

The amount of D234 was measured in an IgM ELISA with D234 as a standard. However, 10 mg of D234 by ELISA equals 14 mg by standard Biuret assay with BSA as a standard. The non-specific control, Rockland human IgM, was given at an equal protein dose by Biuret; thus, 14 mg/mouse.

In the E. coli SM18 infection model, mice administered D234 at 10 mg/mouse showed 60% survival as compared to 30% for the non-specific antibody control (i.e., Rockland IgM) (Table VIII-4). This difference is at the $P = 0.06$ level, which is indicative of a
5 biologically meaningful difference. Thus, it is likely that the protective effect of D234 in the experimental E. coli sepsis model is associated with the administration of core LPS binding monoclonal antibody.

TABLE VII-1

In vivo Effect of D234

| <u>E. coli SM18 Infection Model</u> | | % Survival | |
|--|--|--------------|--------------|
| <u>Test</u> | | <u>3 day</u> | <u>7 day</u> |
| 5 Bacteria control | | 5 | 5 |
| Gentamicin + type specific antibody (2 mg) | | 85 | 85 |
| Gentamicin + non-specific antibody (2 mg) | | 30 | 30 |
| 10 Gentamicin + D234 (10 mg) | | 75 | 65 |
| Gentamicin alone | | 40 | 40 |
| (20 mice per group) | | | |

$P = 0.004$ (between 30 and 75)
 $P = 0.025$ (between 40 and 75)
 $P < 0.03$ (between 30 and 65)
 $P < 0.1$ (between 40 and 65)

TABLE VIII-2

In vivo Effect of D234 - Evaluation of Dose Response

| <u>E. coli SM18 infection model</u> | | % Survival | |
|-------------------------------------|--|---------------|-----------------|
| <u>Test</u> | | <u>3 day</u> | <u>7 day</u> |
| Bacterial control | | 0 | 0 |
| Gentamicin + type specific antibody | | 80 | 80 (P = 0.001)* |
| Gentamicin + D234 (10 mg) | | 70 | 70 (P = 0.01) |
| 20 Gentamicin + D234 (5 mg) | | 65 (P = 0.03) | 60 (P = 0.057) |
| Gentamicin + D234 (2.5 mg) | | 65 (P = 0.03) | 60 (P = 0.057) |
| Gentamicin alone | | 30 | 30 |
| (20 mice per group) | | | |

* P value, as compared to gentamicin alone control.

25 Chi-Square test

TABLE VIII-3

Evaluation of D234 and Rockland IgM in E. coli
SM18 Infection Model

| <u>Test</u> | <u>% Survival</u> | |
|-------------------------------------|-------------------|--------------|
| | <u>3 day</u> | <u>7 day</u> |
| 5 Bacterial control | 0 | 0 |
| Gentamicin + D234 (2.5 mg)* | 75 | 65 |
| Gentamicin + D234 (1.0 mg)* | 65 | 55 |
| Gentamicin alone | 45 | 40 |
| Gentamicin + Rockland IgM (2.5 mg)* | 70 | 50 |
| 10 (20 mice/group) | | |

*Quantity of IgM injected i.v. per mouse.

TABLE VIII-4

Evaluation of D234 and Rockland IgM in E. coli
SM18 Infection Model

| <u>Test</u> | <u>% Survival</u> | |
|------------------------------------|-------------------|--------------|
| | <u>3 day</u> | <u>7 day</u> |
| Bacteria control | 0 | 0 |
| Gentamicin + D234 (10 mg)* | 65 | 60 |
| Gentamicin + Rockland IgM (10 mg)* | 30 | 30 |
| Gentamicin alone | 25 | 20 |
| 20 (20 mice/group) | | |

*Quantity of IgM injected i.v. per mouse.

TABLE VIII-5

D234 Therapeutic Effect as Expressed as Net Increase in
Percentage Survival of Antibody Treated Groups Over
That of Gentamicin Alone Treated Group

| 5 | Test Antibody | Dose (mg/mouse) | Net Increase in % Survival+ | |
|----|---------------|-----------------|-----------------------------|-----------|
| | | | Day 3 | Day 7 |
| | D234 | 10.0 | 35 (0.03)* | 25 (NS) |
| | | | 40 (0.01) | 40 (0.01) |
| | | | 40 (0.01) | 40 (0.01) |
| 10 | | 5.0 | 35 (0.03) | 30 (0.06) |
| | | 2.5 | 35 (0.03) | 30 (0.06) |
| | | | 30 (0.05) | 25 (NS) |
| | | 1.0 | 20 (NS) | 15 (NS) |
| | Rockland IgM | 10.0 | 5 (NS) | 10 (NS) |
| 15 | | 2.5 | 25 (NS) | 10 (NS) |

+ % survival of antibody treated group minus % survival of Gentamicin alone treated group.

* P value, as compared to Gentamicin alone control.

C. Evaluation of T88 and L118 in E. coli SM18 Injection Model

20 Both T88 and L118 were tested for efficacy in the therapeutic infection model in the same manner as D234. These antibodies were tested at 10 mg/mouse (363 mg/kg) (IgM ELISA) and for T88 also at the lower doses of 2.5, 1.0, and 0.5 mg/mouse. These results are shown in Tables IX-1 and IX-2.

25 Results

By seven days after infection, both T88 and L118 treated mice (at 10 mg/mouse) showed 80% survival as compared to 35% for the mice treated with Gentamicin alone. The level of survival enhancement is highly significant at P less than 0.001. However, at lower
30 antibody dosage, T88 did not show significant protective effect. Lastly, although both T88 and L118 preparations as employed in the

tests were not free of endotoxin, the protective effect observed was not due to endotoxin because these antibodies were administered to mice 30 minutes after infection when endotoxin administration no longer influenced the outcome of infection.

5

TABLE IX-1

In vivo Effect of T88 and L118E. coli SM18 infection model

| | <u>% Survival (7 days)</u> |
|-----------------------------|----------------------------|
| Bacterial control | 0 |
| 10 Gentamicin control | 35 |
| Gentamicin + T88 (10 mg/M) | 80 |
| Gentamicin + L118 (10 mg/M) | 80 |
| (20 mice per group) | |

P = 0.004

TABLE IX-2

15 Evaluation of Dose-Response of T88 in E. coli SM18 Infection Model

| <u>Test</u> | <u>% Survival (Day 7)</u> |
|-------------------------------------|---------------------------|
| Bacteria control | 5 |
| Gentamicin + T88 (2.5 mg/m) i.v. | 55* |
| Gentamicin + T88 (1.0 mg/m) i.v. | 40 |
| 20 Gentamicin + T88 (0.5 mg/m) i.v. | 30 |
| Gentamicin alone | 35 |
| Gentamicin + T88 (2.5 mg/m) i.p. | 35 |
| (20 mice/group) | |

* Not significant

Previous work by Nelles and Niswander (1984) Infect. Immun. 46:677-681, and Mutharia et al. (1984) Infect. Immun. 45:631-636, demonstrated that mouse monoclonal antibodies reactive with the Rc-J5 mutant of E. coli exhibited extensive cross-reactivity with other Gram-negative bacterial species. The studies herein demonstrate the feasibility of generating human monoclonal antibodies that react with cross-reactive determinants of the LPS core region, including those related to the biologically reactive lipid A moiety. One or more of these antibodies may be useful as a novel therapeutic agent in Gram-negative sepsis.

These results indicate that human IgM D234 monoclonal antibody that bind to common core determinants on the lipopolysaccharides of Gram-negative bacterial are protective when used in a post-infection treatment mode against lethal Gram-negative bacterial sepsis in mice. The murine model used represents an overwhelming infection challenge in which relatively large doses of monoclonal antibody are needed to induce protection. Mice are known to be comparatively insensitive to endotoxin. For instance, the reported lethal doses of endotoxin in humans are about 100-1000 times smaller (on body weight basis) than that reported for mice. The relevance of endotoxin level is further supported by the fact that in mice dying of E. coli SM18 infection, there is extremely high levels of circulating endotoxin as measured by the LAL assay. Thus, it is likely that in humans, the potential doses of LPS-core reactive monoclonal antibody needed to abate the effects of endotoxin may be much smaller. Thus, human monoclonal antibodies reactive with the common structure of endotoxin may have therapeutic potential for the treatment of life-threatening Gram-negative infections in humans.

D. Evaluation of T88 in Baboons

T88 was shown to be protective against endotoxic shock in baboons. The antibody from one culture supernatant was purified as shown in Figure 3 into two preparations. Both preparations were shown by chromatography on Bakerbond ABx resin, using a salt gradient for elution, to contain two peaks. The T88-10 preparation contained 70%

peak 1 and 30% peak 2, while the T88-10A preparation contained 15% peak 1 and 85% peak 2. The fractionation of the T88 into two peaks was not unique to the Bakerbond ABx resin, but can also be seen with gradient salt elution on other chromatographic matrices such as S-Sephrose and Q-Sephrose. The elution profile for the T88-10A preparation from the Bakerbond ABx column is shown in Figure 4. The T88-10A preparation was used to test for protective activity in baboons.

A total of 5 mg/kg of T88-10A was administered to three baboons. Three mg/kg was administered in a single I.V. bolus 60 minutes before the animals were challenged with a lethal dose of E. coli, and 2 mg/kg simultaneously with the E. coli challenge. About 4×10^{10} organisms were used. The E. coli dose was infused over a two hour period. This dose of E. coli was shown in control experiments to be lethal in 100% of the animals that received it. The animals generally expire within 16 to 32 hours.

Of the three baboons that received T88-10A, one died within the 16 to 32 hour period, and the other two survived. Pathology results suggest that the animal that succumbed may have died for reasons unrelated to the E. coli challenge. These results are consistent with the mouse data reported above, and further establishes that T88-10A is a useful antibody to prevent or to treat septic shock.

E. Characterization of T88 Preparations

The presence of two chromatographic forms in the T88 preparations was surprising, and thus the two preparations from the purification procedure shown in Figure 3 were tested for LPS binding activity as described above. Unexpectedly, the two forms differed dramatically in their LPS binding activity with the T88-10A preparation, which contains predominately the peak 2 form, having strong LPS binding activity, and the T88-10 preparation having predominately the peak 1 form having weak LPS binding activity. Similar work with other LPS binding antibodies (i.e. D234) showed that they also separate into two fractions when chromatographed on the Bakerbond ABx resin as described for T88. SDS gel electrophoresis of both peaks of T8810 or T8810A was indistinguishable, as was an ELISA

for the u chain epitope. Both peaks rechromatographed in their original position when reapplied to the Bakerbond ABx column.

Tables 10 and 11 summarize the T88 u chain assay and LPS binding studies, respectively, for the peak 1 and 2 antibody seen in the two separate purification runs of T88, that is T8810 and T8810A. In Table X, $\sum pk(1+2)$ represents the total amount of antibody present in peaks 1 and 2. "u" represents the amount of u chain to yield an O.D. of 1. $1/ng$ is the reciprocal of the concentration of antibody required to yield an O.D. of 1. $\sum/(1/ng)$ is the ratio of the total amount of antibody, and the reciprocal of the concentration needed to yield an O.D. of 1. It is clear from the data that the amount of u chain is nearly identical for both peaks of either T8810 or T8810A.

Table X

| | <u>Sample</u> | <u>Pk(1+2)</u> | <u>u.(ng)</u> | <u>1/ng</u> | <u>$\sum/(1/ng)$</u> |
|----|---------------|----------------|---------------|-------------|---------------------------------|
| 15 | T88-10 | 1.184 | 25.3 | 0.0395 | 29.96 |
| | T88-10A | 1.726 | 15.0 | 0.06667 | 25.89 |

Table XI compares the areas of peaks 1 and 2 to LPS binding activity measured by ELISA. The areas of peak 1 and 2 are represented by pk1 and pk2, respectively. ng.LPS represents the amount of LPS required to produce an OD of 1. Similarly $1/ng.LPS$ is the reciprocal of the LPS concentration required to give an OD of 1. $pk2/(1/ng)$ is the ratio of the amount of peak 2 antibody to the amount of the reciprocal of LPS required to give an OD of 1. It is apparent from the data that peak 2 exhibits enhanced LPS binding activity.

25

Table XI

| <u>Sample</u> | <u>Pk #1</u> | <u>Pk #2</u> | <u>ng.LPS</u> | <u>1/ng.LPS</u> | <u>$Pk2/(1/ng)$</u> |
|---------------|--------------|--------------|---------------|-----------------|--------------------------------|
| T88-10 | 0.776 | 0.408 | 16.2 | 0.0617 | 6.61 |
| T8810A | 0.338 | 1.387 | 5.46 | 0.183 | 7.57 |

Of the hybrids described herein, D234, D267, L118, the 'W' series of produced antibodies, and T88 were considered the best and were deposited in and accepted by the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland, USA under the terms of the Budapest Treaty. Deposit dates and accession numbers are given below:

| | <u>Hybridoma</u> | <u>Deposit Date</u> | <u>Accession No.</u> |
|----|------------------|---------------------|----------------------|
| | 234-4-27-8 | August 10, 1984 | HB8598 |
| | 267-22-49 | August 23, 1984 | HB8607 |
| 10 | WI-3A | February 28, 1985 | HB8735 |
| | WI-4A | February 28, 1985 | HB8733 |
| | WI-5A | February 28, 1985 | HB8736 |
| | WI-6D | February 28, 1985 | HB8734 |
| | T-88 | May 19, 1987 | HB9431 |
| 15 | L-118 | May 19, 1987 | HB9430 |

In addition, the mouse x human fusion partner F3B6 adapted to 99% serum-free medium which partner was the source of these hybridomas was deposited with the ATCC, with the deposit date and accession number given below:

| | <u>Fusion Partner</u> | <u>Deposit Date</u> | <u>Accession No.</u> |
|----|-----------------------|---------------------|----------------------|
| 20 | F3B6 | April 18, 1985 | HB8785 |

The deposits above were made pursuant to a contract between the ATCC and the assignee of this patent application, Cetus Corporation. The contract with ATCC provides for permanent availability of the progeny of these cell lines to the public on the issuance of the U.S. patent describing and identifying the deposit or the publications or upon the laying open to the public of any U.S. or foreign patent application, whichever comes first, and for availability of the progeny of these cell lines to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 USC .122 and the Commissioner's rules pursuant thereto

(including 37 CFR .1.14 with particular reference to 886 OG 638). The assignee of the present application has agreed that if the cell lines on deposit should die or be lost or destroyed when cultivated under suitable conditions, they will be promptly replaced on notification
5 with a viable culture of the same cell line.

A number of modes for carrying out the invention are contemplated as within the scope of the invention. Thus, although the examples above describing therapeutic modalities using anti-J5 monoclonal antibodies and Gentamicin are described, other broad
10 spectrum antibiotics are effective in controlling systemic Gram-negative bacteremia. In general, Gram-negative bacteremias are treated with aminoglycoside antibiotics and an additional antibiotic such as the cephalosporins, penicillins, beta-lactams, chloramphenicol, erythromycin, vancomycin, trimethoprim sulfa,
15 clindamycin, rifampin, metronidazole and the quinolone antibiotics. Thus, the invention is considered to encompass a mode of therapy in which human anti-LPS antibody is administered to a subject alone or before, after or simultaneous with the administration of antibiotics used for treatment of Gram-negative bacteremia.

Furthermore, the route of administration of antibiotic to the subject in need thereof may be varied depending upon the generally preferred route of administration of any particular antibiotic and the clinical status of the subject in need of treatment. Thus, antibiotic may be administered i.m., i.v., i.p. or if required, intrathecally
20 into the cerebrospinal fluid. Antibody will be administered i.v. or i.p., but most preferred is i.v. Antibody and antibiotic may be coadministered if the pharmaceutically acceptable carriers are compatible.

Without being bound by the theory, it is believed that human
30 anti-LPS monoclonal antibodies affect endotoxic shock associated with sepsis caused by Gram-negative bacteremia by either (1) enhanced host clearance of whole bacteria infecting the host, thereby reducing the source of endotoxin, and (2) enhanced host clearance and subsequent

detoxification of endotoxin by the reticuloendothelial system and liver.

Modifications of the above-described modes for carrying out the invention that are obvious to those of skill in the fields of hybridoma technology, immunology, bacterial infections, and related fields are intended to be within the scope of the following claims.

WHAT IS CLAIMED IS:

1. An antibody characterized in that
 - (a) its population is substantially homogeneous;
 - (b) it binds strongly to determinants that are defined by
5 the lipid A of the cell wall lipopolysaccharides of either E. coli Rc mutants or Salmonella Re mutants;
 - (c) it binds to either the E. coli Rc mutant lipid A determinants or the Salmonella Re mutant lipid A determinants in intact LPS and in whole Gram-negative bacteria; and
 - 10 (d) it blocks the adverse biological effects of Gram-negative bacteria endotoxin.
2. The antibody of claim 1 wherein the antibody is an IgM.
3. The antibody of claim 2 wherein the antibody is of human or rat origin.
- 15 4. The antibody of claim 1 wherein the antibody is selected from the group consisting of the antibodies produced by hybridomas HB8598, HB8607, HB8735, HB8733, HB8736, HB8734, HB9430, HB9431, and functional equivalents of said antibodies.
5. A stable, permanent hybrid cell line that produces the
20 antibody of claim 1 and progeny thereof.
6. The hybrid cell line of claim 5 wherein the hybrid cell line is selected from the group consisting of HB8598, HB8607, HB8735, HB8733, HB8736, HB8734, HB9430, and HB9431.
7. A composition for treating bacteremia or sepsis
25 comprising a therapeutically effective amount of the antibody of claim 1 in association with a pharmaceutically acceptable parenteral vehicle.

8. A composition for treating bacteremia or sepsis comprising a therapeutically effective amount of a plurality of distinct antibodies of claim 1, each of said antibodies binding to a different determinant in association with a pharmaceutically acceptable parenteral vehicle.

9. A composition for treating bacteremia or sepsis comprising a therapeutically effective amount of the antibody of claim 4 in association with a pharmaceutically acceptable parenteral vehicle.

10. A method for treating a mammalian patient for Gram-negative bacteremia or sepsis comprising administering an effective amount of the antibody of claim 1 to the patient parenterally.

11. A method for treating a mammalian patient for Gram-negative bacteremia or sepsis comprising administering an effective amount of the composition of claim 7 to the patient parenterally.

12. A method for treating a mammalian patient for Gram-negative bacteremia or sepsis comprising administering an effective amount of the composition of claim 8 to the patient parenterally.

13. A method for treating a mammalian patient for Gram-negative bacteremia or sepsis comprising administering an effective amount of the composition of claim 9 to the patient parenterally.

14. A stable mouse x human fusion partner consisting of HB 8785.

15. A method according to claim 10 wherein said antibody is administered to the patient before, after, or while administering antibiotics effective in treating Gram-negative bacteremia or sepsis.

16. A method according to claim 7 wherein said antibody is administered to the patient before, after, or while administering antibiotics effective in treating Gram-negative bacteremia or sepsis.

17. A method according to claim 8 wherein said antibody is administered to the patient before, after, or while administering antibiotics effective in treating Gram-negative bacteremia or sepsis.

18. A method according to claim 9 wherein said antibody is administered to the patient before, after, or while administering antibiotics effective in treating Gram-negative bacteremia or sepsis.

19. A method for separating an antibody preparation into two fractions, fraction 1 and fraction 2, that have differential lipopolysaccharide binding activities, comprising the steps of:

(a) absorbing said preparation to a hydrophobic column and eluting said preparation;

(b) contacting said eluted preparation to a first cation exchange resin, and eluting said preparation; and

(c) contacting said eluted preparation from said first cation exchange resin to a second cation exchange resin, and eluting said preparation with an effective linear salt gradient into said two fractions.

20. The method as described in claim 19, wherein said hydrophobic column is phenyl sepharose.

21. The method as described in claim 20 wherein said first cation exchange column is Q-Sepharose.

22. The method as described in claim 21 wherein said second cation exchange column comprises Baker Bond AB_x resin.

23. Antibody present in peak 1 of claim 19.

24. Antibody present in peak 2 of claim 19.

FIG. 1

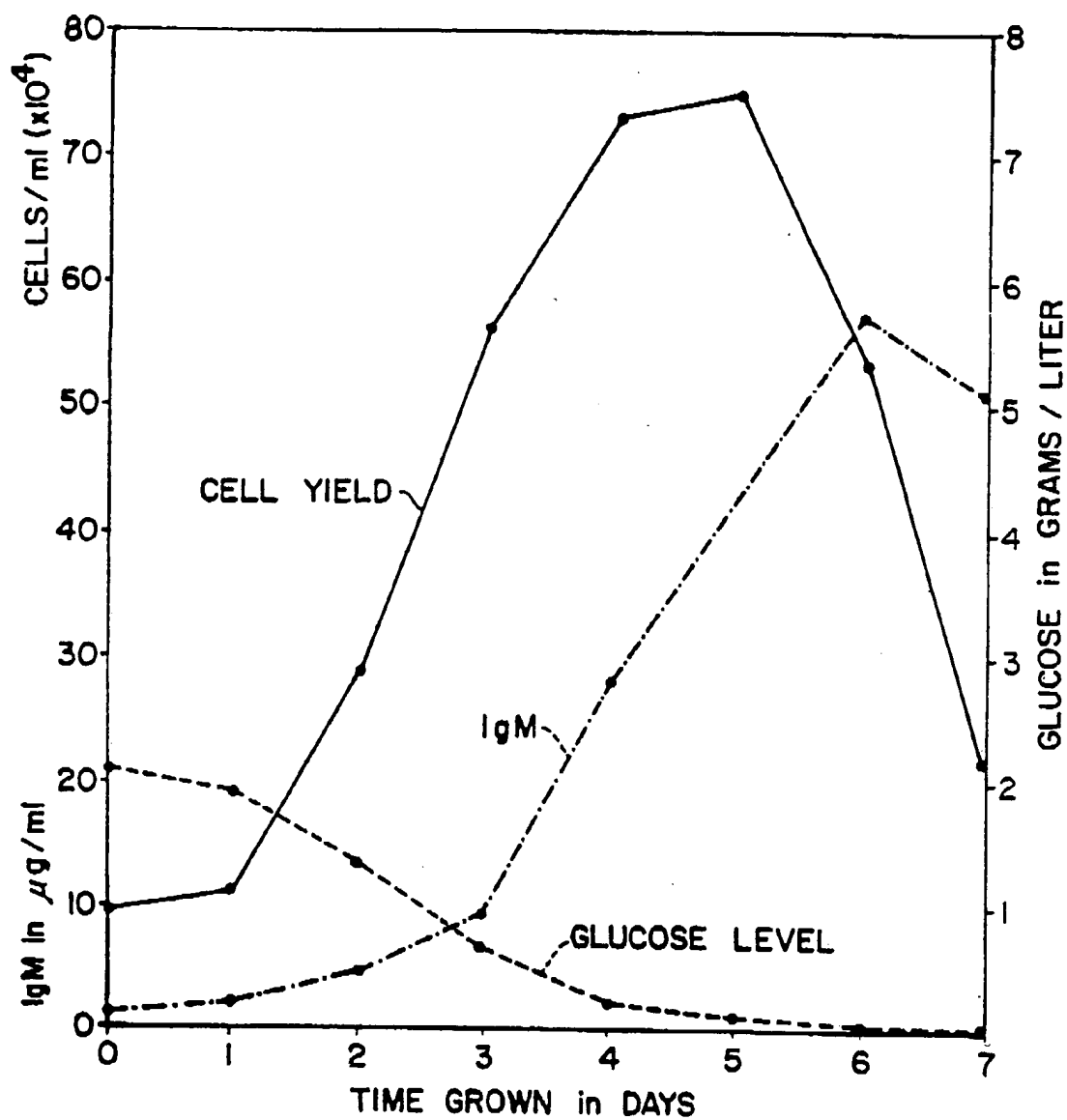
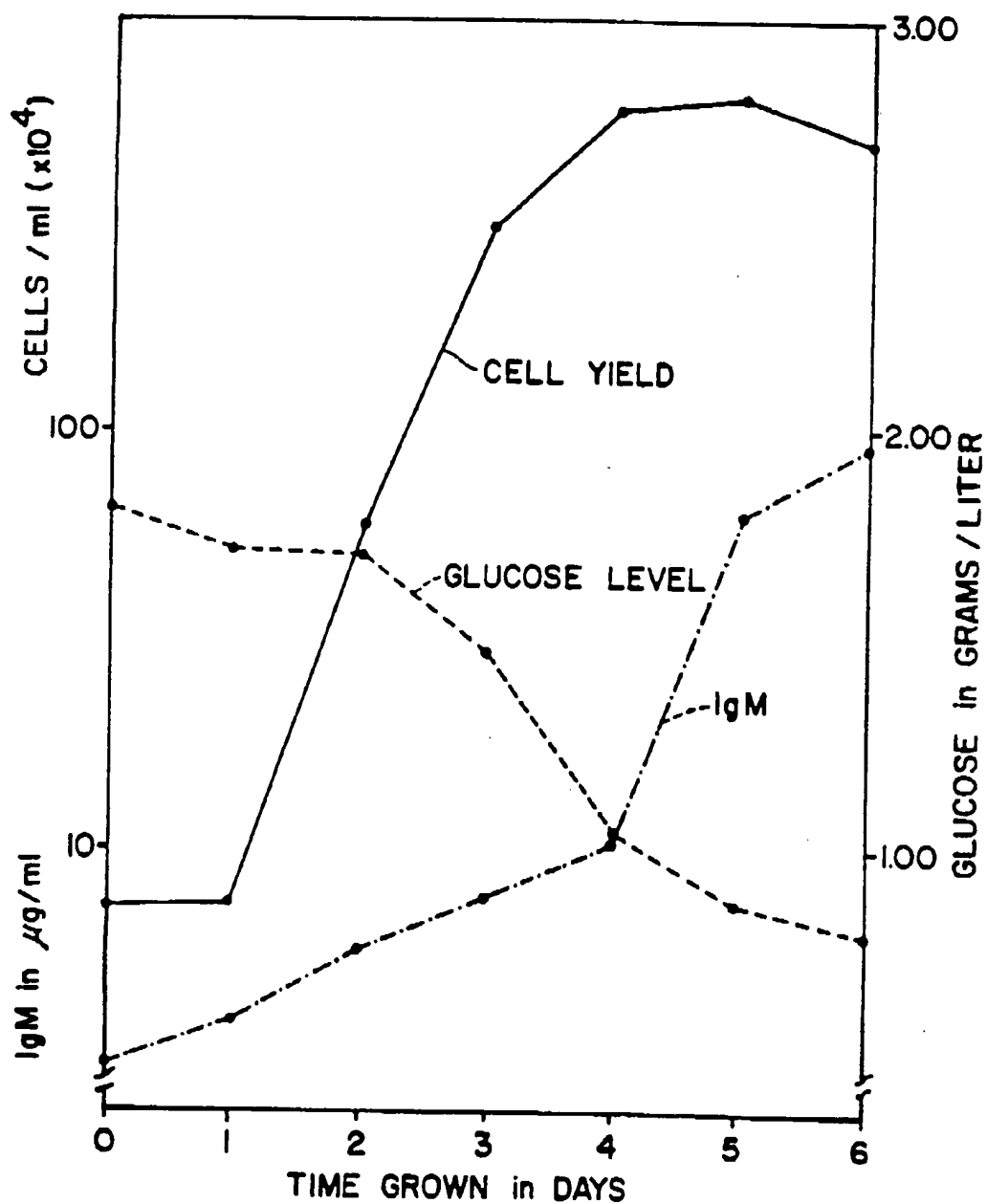


FIG. 2



3/4

FIG. 3

Impure MAB - T88 solution



Add EDTA to 1 mM

Add 100g PEG-8000 per liter of solution



Collect precipitate by filtration



Wash precipitate with PEG buffer

100g PEG-8000 per liter of 10 mM NaPO_4H 7.5 + 150 mM NaClRedissolve precipitate with 10 mM NaPO_4 pH 7.5 + 150 mM NaCl

Add solid NaCl to bring NaCl to 1.6 M



Purify by chromatography

Phenyl Sepharose Fast Flow Column

(step gradient 1.6 M NaCl \longrightarrow 0.3 M NaCl/35% ethylene glycol
pH 7.5) in 10 mM NaPO_4 

In-Process Filtration (0.2 u filter)

Dilution with buffer to 10 mM NaPO_4 pH 8.5 + 160 mM NaCl

Purify by Chromatography Q Sepharose Fast Flow

Step gradient 0.16 M \longrightarrow 0.4 M NaCl in 10 mM NaPO_4 pH 8.5

Elution material is T88-10



Flow Through from Loading Q Sepharose Fast Flow Column

Dilution with buffer to 10 mM NaPO_4 pH 8.5 + 10 mM NaCl

Purify by Chromatography Q Sepharose Fast Flow

Step gradient (0.11 M NaCl \longrightarrow 0.40 M NaCl in 10 mM NaPO_4 pH 8.5

Elution Material is T88-10A

SUBSTITUTE SHEET

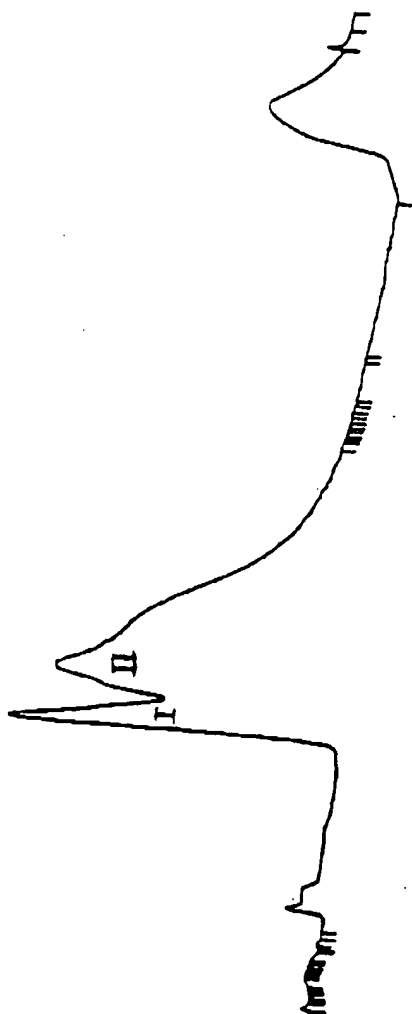
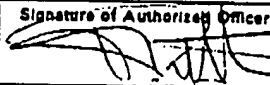


FIG. 4

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 88/03211

| | | |
|---|---|---|
| I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) * According to International Patent Classification (IPC) or to both National Classification and IPC IPC ⁴ : A 61 K 39/40, C 07 K 15/00, C 12 N 5/00, C 07 K 3/20, C 12 P 21/00, //C 12 N 15/00, (C 12 P 21/00, C 12 R:91) | | |
| II. FIELDS SEARCHED <div style="display: flex; justify-content: space-between;"> <div style="width: 30%;"> Classification System </div> <div style="width: 70%;"> Minimum Documentation Searched † Classification Symbols </div> </div> <div style="display: flex; justify-content: space-between; margin-top: 10px;"> <div style="width: 30%;">IPC⁴</div> <div style="width: 70%;">A 61 K, C 12 P, C 12 N, C 07 K</div> </div> <div style="text-align: center; margin-top: 10px; font-size: small;"> Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched * </div> | | |
| III. DOCUMENTS CONSIDERED TO BE RELEVANT* | | |
| Category * | Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹² | Relevant to Claim No. ¹³ |
| X | EP, A, 0174204 (CETUS CORP.) 12 March 1986 see the whole document | 1-9, 14 |
| Y | -- | 19-24 |
| Y | L.B. Schook: "Monoclonal Antibody Production Techniques and Applications", Immunology series, vol. 33, 1987, M. Dekker (New York, US), L.J. Crane: "Purification of mono- clonal antibodies by high-performance ion-exchange chromatography", pages 139-171, chapter 9 see particularly pages 142, 160-163 ----- | 19-24 |
| <div style="display: flex; justify-content: space-between; font-size: x-small;"> <div style="width: 45%;"> <p>* Special categories of cited documents: 10</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p> </div> </div> | | |
| IV. CERTIFICATION | | |
| Date of the Actual Completion of the International Search 29th June 1989 | | Date of Mailing of this International Search Report 31 JUL 1989 |
| International Searching Authority EUROPEAN PATENT OFFICE | | Signature of Authorized Officer  P.C.G. VAN DER PUTTEN |

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE :

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☒ Claim numbers because they relate to subject matter not required to be searched by this Authority, namely:
 - * Claims 10-13, 15-18 Methods for treatment of the human or animal body by surgery or therapy, as well as diagnostics methods.
 - See PCT Rule 39.1 (iv)

2. ☐ Claim numbers because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(e).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING :

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

US 8803211

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 25/07/89. The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

FILED FORN: P0479

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82